Racial Difference in Human Platelet PAR4 Reactivity Reflects Expression of PCTP and miR-376c

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Racial Difference in Human Platelet PAR4 Reactivity Reflects Expression of *PCTP* and *miR-376c*

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Abstract

Racial differences in the pathophysiology of atherothrombosis are poorly understood. We explored the function and transcriptome of platelets in healthy black (n = 70) and white (n = 84) subjects. PAR4 thrombin receptor induced platelet aggregation and calcium mobilization were significantly greater in black subjects. Numerous differentially expressed (DE) RNAs were associated with both race and PAR4 reactivity, including phosphatidylcholine transfer protein (*PCTP*), and platelets from blacks expressed higher levels of PC-TP protein. PC-TP inhibition or depletion blocked activation of platelets or megakaryocytic cell lines through PAR4 but not PAR1. *MiR-376c* levels were DE by race and PAR4 reactivity, and were inversely correlated with *PCTP* mRNA levels, PC-TP protein levels and PAR4 reactivity. *MiR-376c* regulated expression of PC-TP in human megakaryocytes. A disproportionately high number of miRNAs DE by race and PAR4 reactivity, including *miR-376c*, are encoded in the *DLK1-DIO3* locus, and were lower in platelets from blacks. These results support PC-TP as a regulator of the racial difference in PAR4-mediated platelet activation, indicate a genomic contribution to platelet function that differs by race, and emphasize a need to consider race effects when developing anti-thrombotic drugs.

Myocardial infarction and other ischemic arterial diseases like stroke typically result from an occlusive platelet thrombus formed at the site of a ruptured or eroded atherosclerotic

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Author Contributions

PFB conceived the PRAX1 study. PFB and CS designed the PRAX1 study. PFB, LCE, JD, CS, and SN supervised the project. LCE, LMS, ESC, RT, MH, DC, JD, CS, and PFB designed experiments. LCE, LMS, ESC, AB, XK, RT, and MH collected data. LCE, LMS, ESC, PY, RT, MH, JD, CS, and PFB analyzed data. LCE, EC, DC, CS, and PFB wrote the manuscript.

plaque[1]. Thrombin is an especially potent physiologic agonist mediating *in vivo* platelet activation, and human platelets express two thrombin receptors, protease activated receptors 1 and 4, referred to as PAR1 and PAR4[2], both of which mediate thrombin signaling in platelet activation. During thrombin-induced platelet activation these receptors couple to specific G proteins, leading to activation of phospholipases and protein kinases, hydrolysis of phosphoinositides and increased cytoplasmic calcium[3]. Numerous differences in platelet activation have been characterized following stimulation of PAR1 or PAR4[4–7]. For example, compared to PAR1, PAR4 induces a more sustained rise in $[Ca^{2+}]_I$[7] and is responsible for the majority of intracellular calcium flux. These observations suggest different kinetics or signaling pathways through platelet PAR1 and PAR4.

There is reproducible variation in platelet reactivity among different individuals – a variation that likely contributes to thrombotic risk. The inter-individual variation in platelet reactivity is heritable[8], and this heritability is greater in blacks than in whites[9], but there is limited understanding of the genetic mechanisms responsible for this variability. Race is an independent predictor of survival in coronary heart disease even when demographic, socioeconomic, and clinical factors are considered[10,11] suggesting there are yet-to-be identified factors accounting for this racial disparity. We hypothesized a difference in platelet function may represent an important mechanism accounting for some of the racial disparity in thrombotic risk. Human platelets offer a unique opportunity to assess the functional genomics of a primary cell in a relatively non-invasive and high-throughput manner because they can be obtained by sampling the peripheral blood. We designed the Platelet RNA And eXpression-1 (PRAX1) study to identify novel mRNAs and miRNAs responsible for inter-individual variation in platelet reactivity using a cohort of 154 black or white healthy individuals. We discovered racial differences in platelet function and gene expression patterns that appear to contribute to this variation.

Results

Platelets from blacks demonstrate enhanced aggregation through PAR4

We performed *ex vivo* platelet aggregation testing on 163 young, non-diabetic and generally healthy subjects. After exclusion due to use of anti-platelet medication or abnormal hematological parameters, we included 154 subjects for RNA profiling and analyses (Supplementary Table 1). When comparing platelet function from the 70 blacks and 84 whites, we observed no racial difference in the average platelet maximal aggregation response to arachidonic acid, ADP, anti-CD9 antibody, collagen-related peptide or the PAR1 activation peptide (PAR1-AP), which activate platelets through the thromboxane, P2Y1/P2Y12, FcγRIIa, glycoprotein VI and PAR1 signaling receptors, respectively (Fig. 1a). However, aggregation in response to PAR4-AP, which activates platelets through the PAR4 thrombin receptor, was higher in platelets from blacks compared to white subjects (3.8-fold higher at 50 μM PAR4-AP [P<0.0001] and 1.4-fold higher at 75 μM PAR4-AP [$P < 0.0001$]) (Fig. 1a; Supplementary Table 2). Using an agonist response score (ARS) that allowed precise differentiation among subjects with the same maximal aggregation (defined in Methods), the racial difference in PAR4-mediated platelet aggregation was even stronger ($P=6.76\times10^{-9}$). Race was the dominant determinant of the PAR4 ARS when we considered the racial differences in age, gender, body mass index (BMI) and platelet count (Supplementary Table 1) in a multiple linear regression analysis ($P=5.15\times10^{-8}$).

To address the possibility that PAR4-AP did not reflect the true thrombin response, we performed a detailed dose-response study (4 blacks and 3 whites) for racial differences in the platelet aggregation response to thrombin, the physiologic agonist of PAR4 and PAR1 (Supplementary Fig. 1a). When we restricted thrombin signaling to PAR4 by inhibiting PAR1 with BMS-200261[12], platelets from black subjects aggregated faster than platelets

from white subjects at low concentrations of thrombin (Supplementary Fig. 1b–e). We observed no racial difference in maximal percent aggregation to high concentrations of thrombin through PAR4 (Supplementary Fig. 1a–d). Guided by results of the detailed thrombin dose-response study, we performed a replicate study with an additional 5 black and 5 white subjects. At low doses of thrombin (0.6 nM – 1 nM) platelets from black subjects again aggregated faster than white subjects in the absence of PAR1 signaling (P=3.56×10^{-5}) (Fig. 1b).

Principal components analysis applied to genome-wide genotyping data from PRAX1 subjects revealed two distinct and non-overlapping groups that accounted for the largest variance. Superimposition of the self-identified race information onto the PCA results (Fig. 1c) indicated a 100% concordance. Taken together, these data indicate a racial difference specific to PAR4 signaling in human platelets.

Platelet protein coding transcripts DE by PAR4 reactivity and race

We prepared highly purified, leukocyte-depleted platelets (LDPs) from the PRAX1 study subjects by density centrifugation of citrated whole blood followed by immunodepletion of CD45+ leukocytes[13]. We profiled LDP mRNA by the Human Gene 1.0 ST Array (Affymetrix). Based on a probability density plot, we selected a threshold for expression (Supplementary Fig. 2, left panel).

Among expressed transcripts reflecting more than 9000 commonly expressed annotated human genes, we identified 113 as DE by PAR4 reactivity, of which 93 correlated positively (Supplementary Table 3a) and 20 correlated negatively (Supplementary Table 3b), using a False Discovery Rate (FDR) q-value cutoff of 0.25[14]. We found that 97 of 113 (86%) of the PAR4 correlated transcripts were also DE between races, a much higher fraction than would be expected from the background rate of 30% DE by race among all platelet expressed transcripts (OR=14.08, 95% CI.8.23–25.65, P=2.58×10^{-34}), or the 36% DE by race found by Zhang et al[15]. We performed analyses to ensure that racially divergent SNVs occurring in the probe sequences on the Affymetrix Human Gene 1.0 ST Array did not drive the observed probe-set level (transcript/gene) correlations (Supplementary Fig. 3).

Phosphatidylcholine transfer protein (PC-TP) and PAR4 reactivity

Since some of the 97 transcripts that were DE by race and PAR4 reactivity would be expected to be false positives, we performed Gene Ontology (GO) analysis utilizing the Database for Annotation Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp) as a guide for considering the most plausible true associations. Using default settings and all assayed mRNAs as the reference, we found mRNAs DE by PAR4 reactivity to be positively enriched in genes with phospholipid transporter activity (P=8.0×10^{-3}), serine/threonine kinases (P=4.4×10^{-2}) and proteins with pleckstrin homology (P=2.9×10^{-3}). All of these functional categories are known to be relevant to platelet physiology. The gene encoding PC-TP (also called StARD2)[16], *PCTP*, showed the strongest correlation with race (P=10^{-23}; q=10^{-20}) and with PAR4 reactivity (P=3.4×10^{-8}; q=3.5×10^{-4}) (Supplementary Table 3a). This was intriguing because lipid regulation is important for platelet function, and PC-TP is highly specific for binding phosphatidylcholine (PC) and catalyzes its transfer between membranes *in vitro*[16]. Blacks expressed *PCTP* mRNA approximately 4-fold higher than whites (Fig. 2a), and we confirmed this result by qRT-PCR (Fig. 2b) using samples selected to represent the extremes of the distribution as well as intermediate levels. The Spearman Rank correlation coefficient between the microarray and PCR data was 0.8263, and is within the normal validation range when comparing microarray and qRT-PCR data[17]. We also validated this racial difference by western immunoblotting of platelet lysates from all 70 black and 84 white subjects (Fig.

2c & 2d). Finally, we found a significant correlation between the normalized PC-TP protein levels and reactivity to PAR4-AP among the PRAX1 samples (r=0.249, *P*=0.002, Pearson correlation).

Pctp has been knocked out in mice[18], but we found that wild type mouse platelets express little or no Pctp protein (Fig. 2e), consistent with mouse platelet RNA data from Rowley et al[19]. Thus, we needed other approaches to test its function in human platelets. Compound A1 (LDN-193,188), which has been identified as a small molecule inhibitor of the PC transfer activity of PC-TP[20], displaces PC from the lipid binding pocket and increases the thermal stability of the protein[21]. Incubation of human platelets with this specific PC-TP inhibitor prevented aggregation in response to PAR4-AP but not PAR1-AP (Fig. 2f). Calcium mobilization is a critical signaling event upon platelet activation through PAR4. The megakaryocytic cell line, Meg-01 mobilizes calcium in response to thrombin [22], and similar to platelets, we observed the PAR4-mediated intracellular calcium concentration was more sustained than was the PAR1 response. Knockdown of PC-TP using siRNA blunted calcium release in response to PAR4-AP but not PAR1-AP (Fig. 2g–h). In addition, compared to platelets from white subjects, platelets from black subjects showed greater calcium mobilization in response to PAR4-AP (Fig. 2i). These data demonstrate a role for PC-TP in PAR4-mediated calcium flux in platelets and are consistent with PC-TP mediated racial differences in platelet aggregation.

The *PCTP* gene is targeted by *miR-376c*

MicroRNA (miRNA)-mediated mRNA degradation represents a potential mechanism for altering mRNA levels[23,24]. Prior efforts at platelet miRNA profiling have been rather small and lacked data for PAR-mediated platelet activation[13,25,26]. We profiled the 154 PRAX1 platelet samples for miRNA levels and identified 178 miRNAs as commonly expressed in human platelets using threshold criteria similar to the approach used for mRNA (Supplementary Fig. 2). We considered the common platelet miRNAs that are DE by race and predicted to target mRNAs also DE by race and PAR4 reactivity. Figure 3a concisely presents the patterns observed in our data: (i) PAR4 response is higher in blacks than whites (Fig. 3a, upper heatmap), (ii) corresponding elevation in blacks of those mRNAs that positively correlate with the PAR4 response (Fig. 3a, middle heatmap shows more yellow in left half) and (iii) miRNAs negatively associated with PAR4 reactivity that show lower expression in blacks (Fig. 3a, more blue in lower left heatmap). Figure 3b illustrates a focused network of miRNA-mRNA pairs in which 1) both the miRNA and mRNA are DE by race, 2) both the mRNA and miRNA correlate with PAR4-mediated platelet reactivity, 3) the miRNA is predicted to target the mRNA, and 4) the miRNA and mRNA levels are inversely correlated.

Our analysis predicted *PCTP* to be targeted by *miR-376c* (Fig. 3b), and *miR-376c* levels significantly inversely correlated with *PCTP* mRNA levels, PC-TP protein levels, and PAR4 reactivity in the PRAX1 cohort (Table 1). Over-expression of *miR-376c* reduced PC-TP protein expression in HCT cells (Fig. 4a) as well as the Meg-01 megakaryocytic cell line (Fig. 4b), and reduced *PCTP* mRNA levels in Meg-01 cells and the HEL megakaryocyte cell line (Fig. 4c). Additionally, inhibition of *miR-376c* caused *PCTP* mRNA levels to rise (Fig. 4c). We then isolated CD34+ hematopoietic stem cells from human cord blood and differentiated them into megakaryocytes and proplatelets (Fig. 4d–e). Transfection of *miR-376c* LNA inhibitor into CD34+-derived CD61+ megakaryocytes increased *PCTP* mRNA levels by more than 3-fold, while overexpression of the *miR-376c* precursor decreased *PCTP* mRNA by 22% (Fig. 4f). The extent of *miR-376c* knockdown by the anti-*miR-376c* LNA inhibitor and overexpression by the precursor is shown in Supplementary Fig. 4. Transfection with the LNA inhibitor had no effect on the expression level of a

different *miR-376* family member, *miR-376b* or on other miRNAs located within the same miRNA cluster, *miR-410* and *miR-495* (Supplementary Fig 4). Finally, reporter gene assays demonstrated the *direct* action of *miR-376c* on the wild type *PCTP* 3′UTR but not a mutant *PCTP* 3′UTR construct with a deleted *miR-376c* binding site (Fig. 4g).

The *DLK1-DIO3* miRNA cluster is expressed higher in whites

MiR-376c is one of a set of miRNAs DE by race that appeared to be positively correlated with one another (Fig. 3a, vertical blue and yellow swatches in the miRNA heatmap; highlighted by the vertical red bar next to the miRNA heatmap). Indeed, expression analysis revealed a significant correlation among 24 of the 178 common platelet miRNAs, and all 24 mapped to chromosome 14q32.2 (Fig. 5a). This locus is most commonly called the *DLK1-DIO3* genomic region and contains a large cluster of miRNA genes (Fig. 5b).

When we generated a composite expression score for the miRNAs from this locus for each of the 154 subjects, race showed a strong association with expression (black vs. white $P=1.09\times10^{-5}$). When we included age, gender, BMI and platelet count in a multiple linear regression analysis, race remained the dominant determinant of the *DLK1-DIO3* region miRNA expression ($P=0.000166$, partial F statistic). As shown in Figure 5c, all commonly expressed *DLK1-DIO3* region mature miRNAs ($n=24$) were higher in whites than blacks. Supplementary Table 4 lists the commonly expressed miRNAs DE by race and Supplementary Table 5 lists the *DLK1-DIO3* region miRNAs with mean values, variance and p-values by race. We confirmed racial differences in miRNA expression by qRT-PCR (Supplementary Fig. 5).

Discussion

The richness of the PRAX1 data set provided unique opportunities to establish miRNA-mRNA-physiology relationships that led to a number of novel and unexpected results. Several major findings emerged. First, compared to platelets from whites, platelets from blacks showed greater platelet reactivity in response to activation through the PAR4 thrombin receptor. Since PAR4 is expressed in tissues other than platelets (e.g., heart, brain, liver), these findings could be relevant for non-thrombotic diseases believed to show racial differences. Second, the novel platelet protein, PC-TP, mediated PAR4-dependent calcium mobilization and aggregation. Compared to platelets from whites, platelets from blacks expressed higher levels of PC-TP and greater PAR4-dependent calcium mobilization and aggregation through PC-TP. Third, *miR-376c* regulated expression of PC-TP in both megakaryocytes and megakaryocytic cell lines. We also discovered a genomic module of miRNAs and target mRNAs that strongly correlate with this racial difference in PAR4-mediated platelet aggregation. Many of these miRNAs clustered in the *DLK1-DIO3* region and were expressed at higher levels in platelets from whites than blacks. These findings should be considered in clinical trials involving anti-platelet therapies and on-going drug development of inhibitors of protease activated receptors.

There is a paucity of literature considering racial differences in platelet function, and we are not aware of work considering either thrombin receptors or associations between race and the platelet transcriptome. A single small study reported that calcium mobilization varied by race in patients with hypertension[27], but it was unclear if this difference was due to race or disease (hypertension) and the responsible molecular mechanism was not considered. We found that platelets from blacks demonstrated significantly greater activation to PAR4-AP than platelets from whites. Compared to thrombin, PAR activating peptides have the advantage of discriminating between PAR1 and PAR4 and are amenable to the relatively high-throughput screening needed in the PRAX1 study. Nevertheless, to address the possibility that there could be an unusual interaction between race and PAR4-AP but not

between race and thrombin (i.e., that PAR4-AP in this study does not reflect the true thrombin response), we performed two additional studies with different subjects to assess thrombin-induced platelet aggregation after PAR1 inhibition. Both the thrombin-dose-response study and replicate study showed racial differences mediated by thrombin-activated PAR4. Although the data suggest a possible racial difference in thrombin-induced platelet aggregation in the absence of the PAR1 inhibitor, this difference was less pronounced than in the presence of the PAR1 inhibitor and a larger sample size would be needed to clarify this possibility. Since the greater aggregation in platelets from blacks was apparent only at the earliest time points and lowest thrombin concentrations, the racial difference in PAR4 reactivity may be primarily kinetic. Additional studies are needed to determine whether there is a racial difference in the kinetics of the response to thrombin when both PAR1 and PAR4 are functional. The fact that we observed no racial difference for other agonists strongly supports a relationship between race and PAR4-mediated platelet aggregation, and not the effects of other crucial variables of the read-out such as integrin αIIbβ3 levels or a plasma protein. The data using thrombin and washed platelets also indicate 1) there is not an unknown plasma "cofactor" that differs by race affecting PAR4-mediated platelet activation and 2) the results are not due to racial differences in an undiscovered protease that activates PAR4 and not PAR1. Furthermore, we measured plasma fibrinogen and VWF activity and neither differed by race. Moreover, we observed this racial difference in PAR4-AP induced platelet aggregation in separate cohorts from different cities (Houston and Philadelphia) with independent reagents, instrumentation, and personnel.

A fundamental aspect of our conclusion pertains to race. We recruited black and white subjects randomly throughout the duration of the study and there was no sequential recruitment bias by race. Although self-identified race and ethnicity (SIRE) has been shown to strongly correlate with selected genomic markers and ancient geographical ancestry,[28–31] we validated the use of SIRE in PRAX1 using PCA, which provided an *unbiased* examination of structure in the genotype data. American blacks are an admixed group[32] and African lineages have greater diversity; these facts are reflected in the greater spread within that PCA-defined group. Although this strong relationship between SIRE and genetic markers supported conclusions regarding a genetic effect on platelet function, it does not preclude a contribution of socioeconomic or environmental factors to variation in PAR4 reactivity.

Genome-wide gene expression analysis showed more DE transcripts were positively associated with PAR4 reactivity than were negatively associated. Such asymmetry has been noted in other studies[33]. Perhaps the human platelet response through PAR4 evolved in such a way as to promote blood clotting after trauma, thus providing a survival advantage; genetic drift is another possible explanation. One hundred thirteen mRNAs (out of >9000 expressed transcripts) were DE by both race and PAR4 reactivity. We pursued *PCTP* because of its strong statistical associations and because of its biologic plausibility. PC-TP belongs to the steroidogenic acute regulatory transfer protein-related transfer (START) domain superfamily, which constitutes a functionally diverse group of proteins that share a unique START domain for binding lipids[34]. PC-TP has been presumed to be expressed primarily in the liver, kidneys, and testis. PC-TP protein was not known to be present or function in platelets[35], although *PCTP* mRNA was one of a number of platelet mRNAs whose expression increased after hemodialysis in patients with chronic kidney disease[36]. Besides PC transfer *in vitro*, PC-TP also regulates both insulin and glucose metabolism in mice and human cell lines[16,37,38], but the human *in vivo* function of PC-TP remains unclear. PC comprises a major fraction of platelet phospholipids and multiple subcellular locations of PC may exist[39]. PC is the classic substrate for phospholipase D, generating phosphatidic acid that can be converted to the second messenger diacylglycerol[40]. PC can also be hydrolyzed

by phospholipase C to generate phosphatidylinositol 4,5-bidphosphate with subsequent release of calcium from intracellular stores. The PC-TP inhibitor, compound A1, blocked PAR4- but not PAR1-induced platelet aggregation, supporting of a role for PC-TP in platelet function. While it has been reported that A1 inhibits the closely PC-TP-related proteins StARD7 and StARD10[21], it does so less effectively. Furthermore, *PCTP* mRNA was 7.9-fold and 9.40 fold higher than *STARD7 and STARD10* mRNA respectively, in platelets, according to our microarray data. This suggests that the predominant effect of A1 treatment on platelets was inhibition of PC-TP. The lack of PAR1 inhibition by A1 suggests PC-TP impacts on a signaling event downstream of PAR4 engagement but upstream of pathways substantively shared by PAR1 and PAR4. However, depleting PC-TP in megakaryocytic cells via shRNA interference produced a functional effect on calcium mobilization that was also PAR4- but not PAR1-dependent. These studies in two different experimental systems, in addition to the racial difference seen in PAR4-mediation platelet calcium mobilization (Fig. 2i) support a novel role for PC-TP mediation of intracellular calcium transients and in the racial difference in PAR4-induced platelet activation. The serine-threonine kinase, *AKT3*, was also differentially expressed by race and PAR4 activation, consistent with the observation that *Akt3*−/− murine platelets lack responsiveness to thrombin but not to other agonists[41].

The *DLK1-DIO3* region at the 14q32.2 locus is evolutionarily conserved and imprinted, harboring *DLK1* and *MEG3*, antisense *RTL1*, many small nucleolar RNAs (snoRNAs), long intergenic RNAs (lincRNAs) (including *MEG3*) and the largest currently known cluster of miRNAs in the human genome (reviewed in reference 42). The expression of some of the *DLK1-DIO3* miRNAs has been associated with murine stem cell pluripotency[43], human cancers[42], and paternally inherited type 1 diabetes has been mapped to this region[44]. The molecular mechanism for coordinate expression of human *DLK1-DIO3* region miRNAs is unclear, although data in mice supports processing of a single large transcript containing mature miRNAs 3′ of the snoRNAs cluster[45], and independent genes/transcripts and promoters 5′ of snoRNAs cluster[46]. We note that our data do not exclude a role for lincRNAs or snoRNAs in regulating PAR-dependent gene expression.

Our data demonstrate the miRNAs in this region are expressed at higher levels in platelets from white subjects than black subjects, and it will be important to assess other tissues and the other RNAs encoded in this region for this racial difference. *MiR-376c,* which resides within the *DLK1-DIO3* region, was DE by race and PAR4-induced platelet reactivity and was predicted to target the 3′UTR of *PCTP*. As might be expected if *miR-376c* targeted *PCTP* and resulted in mRNA degradation, *miR-376c* levels were inversely correlated with PC-TP mRNA and protein, and weakly correlated with PAR4-induced platelet aggregation. A series of overexpression, inhibition and binding site mutation studies in a variety of cell types, including primary cultured human megakaryocytes demonstrated direct perturbation of *PCTP* expression by *miR-376c*. Fig. 5d summarizes the proposed relationships identified in this study, emphasizing the race effect on RNAs, leading to racial differences in platelet PAR4 reactivity. Although the there is a strong correlation between the platelet and megakaryocyte transcriptomes[47], racial differences in platelet RNAs cannot be assumed to reflect differences that occurred in the megakaryocytes. Addressing this possibility would require a racially diverse source of progenitor cells. Nevertheless, transcriptomic and bioinformatic analyses of platelet RNA can guide experiments in cells to permit insights into relevant gene regulation in megakaryocytes.

There are clinical consequences to our findings, particularly in an era where personalized medicine has been advanced as a strategy to improve patient outcomes. First, awareness and understanding of differences by which platelets are activated in blacks and whites is expected to aid in our ability to optimally treat these populations following myocardial

infarction and stroke. Although the issue of race serving as a proxy for genetics in medical practice and research is controversial[48], our results suggest SIRE may have value in assessing hemostasis-thrombosis risk in some populations. Anti-platelet therapy is a mainstay of managing coronary heart disease. However, there has been a notable absence or, at best poor representation of blacks in clinical trials of anti-platelet therapies and in genome-wide association studies of atherothrombotic phenotypes. Unfortunately, neither cardiovascular trials with PAR1 inhibitors nor trials listed at clinicaltrials.gov allow assessment of racial effects in clinical outcomes, and it is unknown whether the racial difference in platelet reactivity impacts the benefits and risks of these treatments. The PAR1 inhibitor vorapaxar is effective at preventing secondary atherothrombotic events, but at a cost of increased bleeding[49–51] and other novel inhibitors of PAR1 and PAR4 are currently in clinical development[52]. In the presence of vorapaxar, PAR4 is the only means by which thrombin can activate platelets. Thus, it is critical to know whether selection and dosing of such agents should be adjusted by race to maximize benefit and avoid toxicity. Our findings call for greater access to clinical trial data to address the effect of race on anti-platelet therapies. In addition, the racial differences identified in the *DLK1-DIO3* region miRNA expression may be present in other tissues, and more large scale RNA studies can address this question. Lastly, it will be of interest to know whether PC-TP inhibition might prove useful as an anti-thrombotic strategy, especially in disorders of altered glucose and insulin metabolism.

Online Methods

Subjects

The Institutional Review Boards of Thomas Jefferson University and Baylor College of Medicine approved the Platelet RNA And eXpression-1 (PRAX1) study, and we obtained informed consent from all volunteers. Only healthy, non-diabetic subjects who self-identified their race as white or black, using no anti-platelet medications were eligible for this study. We recruited black and white subjects randomly throughout the duration of the study. We recruited donors from 2010–2011 in Houston, TX, and in 2013 in Philadelphia, PA, and collected blood as previously described[53].

Platelet function assessment

Houston studies. We obtained a complete blood count and mean platelet volume using an ABX Micros 60 CS (Horiba ABX, Irvine, CA, USA). Using light transmission aggregometry, we measured maximal percent aggregation and slope of aggregation at 10 min in platelet rich plasma (PRP) treated with: 0.5 mg ml^{-1} arachidonic acid, 4 μM ADP, 500, 750 and 2000 ng ml^{-1}anti-CD9, 10 and 20 ng ml^{-1} collagen-related peptide (CRP),1 μM and 2 μM PAR1 activating peptide (PAR1-AP), and 50 μM and 75 μM PAR4 activating peptide (PAR4-AP). Philadelphia studies. We used light transmission aggregometry of washed platelets to assess thrombin-induced platelet aggregation without or with a 2 min pre-incubation with 20 μM BMS-200261 (BMS, PAR1-specific antagonist)[12]. We observed a difference in the efficacy of the PAR1 inhibitor between the pilot and replicate studies, but confirmed validation of inhibitor activity for both studies (Fig 1b inset and Supplementary Fig 1e). For assessment of calcium mobilization, we re-calcified platelets to a final concentration of 1 mM followed by pre-incubation with Fluo-4 AM for 10 min. We then stimulated platelets with PAR4-AP and measured calcium mobilization using the Accuri C6 flow cytometer[54]. We minimized, in both Houston and Philadelphia, technical variation by use of the same highly-trained technicians, same lots of reagents and same instruments throughout the duration of the study. Because we used multiple concentrations to activate PAR1 and PAR4 to induce platelet aggregation, we developed an integrated score that combined these values to determine a single index that represented each individual's agonist

response. This score correlated strongly with standard assessment of maximal percent aggregation, but inclusion of slope (which was a minor contributor to the overall score, and highly related to maximal aggregation) allowed distinction among platelets with the same maximal aggregation value. This score was highly reproducible in 10 subjects studied weekly for three or four weeks.

Plasma assays

We measured plasma fibrinogen level and von Willebrand factor activity on an ACL TOP 500 (Instrumentation Laboratories, Bedford, MA) as per manufacturer's instructions.

Cell Lines

We obtained Meg-01 and HEL cells from ATCC (Manassas, VA) where they are authenticated before distribution. R. Baserga kindly provided HCT116-Dicer KO 2 cells (Department of Cancer Biology, Thomas Jefferson University, Philadelphia, PA, USA), and we authenticated these cells for Dicer deficiency. We did not test cell lines for mycoplasma.

Measurement of Ca^{2+} mobilization

24 h before transfection, we plated Meg-01 cells (ATCC, Manassas, VA) at 1×10^6 cells $well^{-1}$ in 6-wells plates with RPMI 1640 media supplemented with 10% FBS without antibiotics. We transfected cells with either human PCTP siRNA-SMART pool or non-targeting siRNA control (ThermoFisher Scientific, Waltham, MA) by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. We harvested cells 48 h after transfection and performed western-blot, qRT-PCR, and flow cytometry assays. For monitoring Ca2+ mobilization under agonist stimulation, cells were washed, pre-incubated with Fluo-4-AM (Invitrogen), recalcified and stimulated with 250 μM PAR4-AP or 50 μM PAR1-AP[54]. We monitored changes in Meg-01 Ca^{2+} concentration over time using an Accuri C6 flow cytometer.

Platelet mRNA profiling

We prepared leukocyte depleted platelets (LDP) RNA by density centrifugation and immune-depletion of CD45+ cells as previously described, yielding less than 1 contaminating leukocyte in 5 million platelets[13]. We extracted RNA with TRIzol® (Life Technologies, Carlsbad, CA). We minimized technical variation by use of the same highly-trained technicians, same lots of reagents and same instruments throughout the duration of the study. All RNA samples were subject to quality control screening using an Agilent Bioanalyzer. We labeled 300 ng of LDP total RNA and hybridized it to the Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). For mRNA data pre-processing, we used "apt-probeset-summarize" from the Affymetrix Power Tools for background subtraction, normalization and summarizing probeset values from Affymetrix Gene array. We adjusted the background with the Robust-Multi-array average technique. We background corrected the raw intensity values, log2 transformed and then quantile normalized them (the command line option was "-a rma-sketch") similar to our prior analysis[55]. Data deposited into NCBI GeneExpressionOmnibus.

Platelet miRNA profiling

We performed digital miRNA profiling from 100 ng of LDP total RNA using the nCounter human miRNA assay kit v1 (Nanostring Technologies, Seattle, WA) that counts miRNAs without amplification and produces high sensitivity and specificity allowing accurate comparisons of individual miRNA species within and between samples. In brief, a color-coded reporter probe and a capture probe are hybridized, in solution to target molecules. The captured molecules are then attached to a streptavidin-coated slide and then elongated and

aligned via electrophoresis. Finally, the color-coded reporter probes are counted. This technology has the advantage over standard microarray hybridization profiling of avoiding the biasing effects of RNA amplification and of direct measurement of the number of molecules detected and demonstrates linearity over 2.5 logs of concentration[56]. Total RNA is hybridized in solution to reporter probes labeled with fluorescent bar codes. Via capture probes, software counted the hybridized miRNAs adhered to a solid surface and the number of bar codes. We determined expression levels of well characterized and validated human (n=654) and human-associated viral miRNAs (n=80). To control for technical differences, we normalized the data using spike-in positive controls. To account for loading differences in miRNA content, we further normalized the data to total number of miRNA counts in each sample. We set the background detection threshold as the average plus two standard deviations of eight negative control probes. Notably, the values obtained in our data fell within the linearity of the Nanostring assay. Data deposited into NCBI GeneExpressionOmnibus.

Validation of RNA expression, and miRNA knockdown

We cultured the HCT-116-Dicer-KO cell line in McCoy's 5A medium (Life Technologies, Carlsbad, CA); and the Meg-01 and HEL cells in RPMI medium (Life Technologies) adjusted to contain 10% (v/v) fetal bovine serum. We seeded cells 24 h before transfection in 6-well plates without antibiotics. To validate *miR-376c* knock down of PCTP, we transfected HCT115-Dicer-KO cells with 20–80 nM of pre-*miR-376c* or scrambled control (Life Technologies). We transfected Meg-01 and HEL cell lines with 100 nM of pre-*miR-376c* or scrambled control (Life Technologies), as well as *hsa-miR-376c* miRCURY LNA™ microRNA inhibitor or miRCURY LNA™ microRNA Inhibitor Negative Control (Exiqon, Denmark) using Lipofectamine LTX (Life Technologies) transfection agent following the manufacturer's protocol. After 6 h we replaced the media. We harvested the HCT-116-Dicer-KO cells 6 h, 24 h and 48 h after transfection. We harvested Meg-01 and HEL cell lines 48 h after transfection. We isolated total RNA using Trizol® Reagent (Ambion). To validate relative expression levels of miRNAs and mRNAs, we performed qRT-PCR of *PCTP* transcripts with the Power SYBR Green PCR master mix (Life Technologies, Carlsbad, CA), using the following primers 5′-AGAATGCAACGGAGAGACTGTGGT-3′, 5′-TCACATGGATCTTCCTCCCTTCCA-3′ and normalized to β-actin. We quantified mature *hsa-miR-376c*, *hsa-miR-376b*, *hsa-miR-410*, *hsa-miR-495* and *let-7b* levels using TaqMan® MicroRNA Reverse Transcription Kit, TaqMan® MicroRNA assays together with TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). We used RNU6B expression to normalize miRNA expression. We carried out real time PCR reaction and analyses in 384-well optical reaction plates using the 7900HT instrument (Applied Biosystems).

Western blotting and antibodies

We lysed leukocyte-depleted platelets and homogenized them in 8 M urea buffer (8 M urea in 10 mM Tris, pH 6.8, 1% SDS, 5 mM DTT in the presence of a 1x protein inhibitor mix (Roche, Indianapolis, IN)). We separated extracts by 12% SDS–PAGE and transferred onto PVDF membranes. We immunostained membranes with goat anti-human PC-TP[57] and mouse anti-human GAPDH monoclonal antibody[58] (Santa Cruz Biotechnology, Dallas, TX). We performed detection and densitometric analysis with Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Hematopoietic Stem Cell (HSC) isolation and differentiation to megakaryocytes

We obtained cord blood from the New York Blood Center and diluted it 1:2 with PBS and layered over Histopaque® (Sigma, St. Louis, MO). After 30 min centrifugation at 400 x g

we carefully collected the buffy coat. We isolated $CD34^+$ cells using magnetic immunoselection (Miltenyi Biotec, Auburn, CA) following the manufacture's protocol. We then cultured cells at 4×10^5 cells $well^{-1}$ in a 24 well plate, with StemSpan™ media (StemCell Technologies, Vancouver, Canada) supplemented with 25 ng mL^{-1} SCF and 20 ng mL^{-1} thrombopoietin (TPO; Peprotech, Rocky Hill, NJ). After 6 d we supplemented the cultures with 50 ng mL^{-1} TPO only. Every 3 d, we harvested the cells, counted and re-plated them at 4×10^5 cells $well^{-1}$ in a 24 well plate with fresh media. We monitored megakaryocytic differentiation by flow cytometry, testing for CD34, CD41 and CD42. At 16 d, we seeded cells onto a fibrinogen coated coverslip. We then stained cells with phalloidin-alexa-533 (Life Technologies), rabbit anti-human tubulin[59] and mouse anti-rabbit alexa-488 (Life Technologies). Then we mounted the coverslips on slides using ProLong® Gold Antifade Reagent with DAPI (Life Technologies). Finally, we captured images using a Zeiss LSM 510 Meta Confocal Laser Scanning Microscope with a META detector.

Statistical analysis

Before performing the study, we performed sample size calculations using a linear model framework with Gaussian errors using effect sizes and variance estimates based on our previous work where we performed genome wide expression profiling with purified platelet samples [13,25]. This effort indicated 150 subjects would provide greater than 85% power to detect modeled associations between platelet agonist phenotypes and RNA expression levels. For continuous phenotype (response) data in this study, we used qq-plots to evaluate normality of errors about linear model fits. For binary count data, we employed binomial sampling models, and used Fisher's Exact tests to evaluate associations, such as when testing the cross tabulation of those genes associated with PAR4 reactivity vs. those associated with self-identified race. We did not find the variance of PAR4 reactivity within each race to be significantly different by an F-test of the variances (F=1.4, 95% CI: 0.87–2.15, P=0.2). We performed statistical analyses using the open source statistical programming environment R[60]. We performed association analyses between platelet phenotype PAR4 and gene expression using linear models. Multiple testing considerations were made using the False Discovery Rate (FDR) methodology, and q-values were estimated using the Benjamini-Hochberg method[61] as made available through the R function *p. adjust*.

Principle components analysis (PCA)—PCA is a commonly used mathematical approach that allows an unbiased examination of population structure in large scale genotype data. PCA transforms high dimensional multimarker genotypes encoded as reference allele counts from many subjects into simple, low dimensional representations through projection to additive combinations of allele counts where the weights are the PCA loadings. This analysis permits identification of "structure" such as race in the data. We applied this unbiased method to our samples. We extracted DNA from buffy coat preparations from all PRAX1 subjects using the Gentra Puregene Blood Kit (Qiagen, Netherlands). We hybridized DNA to the HumanOmni5 array (Illumnia Inc., San Diego, CA) at the Laboratory for Translational Genomics at the Baylor College of Medicine. We used the Eigenstrat software package[62] to compute the PCA transformation excluding the ethnicity information from the analysis.

Filters for detected and background probes—We excluded eight subjects for presumed anti-platelet medication use (defined as arachidonic acid aggregation of <30%) and one subject was excluded because of abnormal hematological parameters, leaving 154 samples for all subsequent analyses. In both the miRNA and mRNA data we used a two-step filter to determine which probes to consider as expressed in our samples for the purposes of downstream analyses. We first used the distribution of mean values for each probe across

subjects to determine a cutoff that differentiated those features with background intensity from others. We then counted the number of individual subjects with values that exceeded this background cutoff. The count distribution for the number of subjects exceeding the background cutoff was bimodal, indicating that most probes are either almost always expressed or not in our cohort. To be considered commonly expressed, we therefore required that at least 100 subjects express the miRNA above background and 115 subjects express the mRNA above background. For the mRNAs we also required that the probeset was of category 'main', as classified in HuGene-1_0-st-v1.na33.2.hg19.transcript.csv file (available from www.affymetrix.com/support/technical/annotationfilesmain.affx). A sensitivity analysis showed very little variation in the selected data based on small variations in the cut-point for expressed RNAs, and the method was also robust to small variations in the percentage cutoff for the number of people expressing each RNA.

Exploratory analyses—We performed exploratory cluster analyses on the miRNA platelet expression data. We centered and hierarchically clustered using Cluster 3.0 normalized log2 transformed values of the 178 miRNAs above cutoff (original software by Michael Eisen[63], updated by Michiel de Hoon (University of Tokyo, http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm)). We created a corresponding heatmap using Java Treeview. We calculated Pearson correlation coefficients for all 178 miRNAs above cutoff pairwise using the R statistical package.

Model based analyses—In addition to cluster analyses we performed a series of correlative analyses to examine the association between RNA expression and subject demographics, platelet agonist-induced aggregation and other hematologic parameters. These analyses included simple and multiple linear regressions, performed in R using analysis of variance (aov) tools that permit evaluation of multiple explanatory variables. In the analysis of the PAR4 phenotype we treated PAR4 reactivity as the dependent variable and included the demographic covariates and RNA expression data as explanatory. We took a False Discovery approach to account for possible false positives in the mRNA expression analysis, and we used the Benjamini-Hochberg linear step down method to control the FDR[14].

Network analysis—We constructed a network of miRNA-mRNA pairs using target predictions and allowing an edge only if miRNA and mRNA levels met the following criteria across the 154 subjects: 1) both the miRNA and mRNA are differentially expressed by race, 2) both the mRNA and miRNA correlate with PAR4-mediated platelet reactivity, 3) the miRNA is predicted to target the mRNA, 4) the RNA levels are inversely correlated with one another, and 5) the miRNAs reside in the *DLK1-DIO3* region. We then visualized the miRNA-mRNA pairs using the Cytoscape software package.

Analysis of Racial SNV Allele Frequency and RNA expression probes—To investigate the possibility that RNA expression differences observed between races could be mediated by SNV variation in the probes in the two expression assays used – Affymetrix Gene ST 1.0 and Nanostring – we performed additional analyses of our expression data using information from the 1000 Genomes project Genomes Project Consortium (A map of human genome variation from population-scale sequencing. Nature. 2010; 467: 1061–73.) as a reference. We obtained data from the 1000 genomes website ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20100804/supporting/, in particular the files:

AFR.2of4intersection_allele_freq.20100804.genotypes.vcf.gz

AFR.2of4intersection_allele_freq.20100804.genotypes.vcf.gz.tbi,

EUR.2of4intersection_allele_freq.20100804.genotypes.vcf.gz

EUR.2of4intersection_allele_freq.20100804.genotypes.vcf.gz.tbi

We then identified all 1 million+ probe sequence locations for probesets on the Affy GeneST 1.0 array and the miRNAs comprising the Nanostring assay. We identified the locations of all SNVs (SNPs) in the thousand genomes data that showed a significant difference in allele frequency between races (EUR and AFR) based on Fisher's exact test of allele frequency and a Fisher's Test p-value cutoff of 0.01, and we identified all those expression probes that harbored a racially dimorphic SNP as defined by the Fisher's test criteria. We then performed analyses to consider their effect on our gene-level expression and miRNA expression results. A boxplot of oligonucleotide probe level data for one of the probesets bearing a probe with a racially dimorphic SNP is presented in Supplementary Figure 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The data discussed in this publication have been deposited in US National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSExxx (Note: data will be deposited upon manuscript acceptance).

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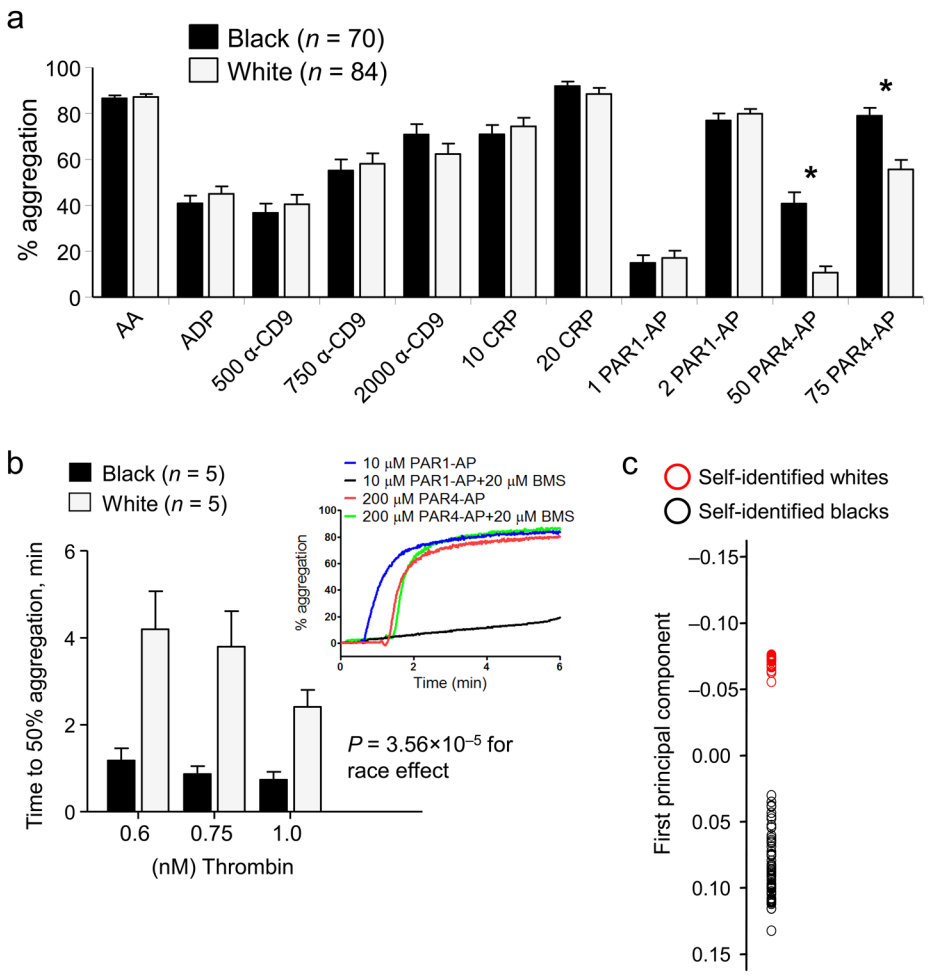

Figure 1.

Racial difference in PAR4-mediated platelet aggregation. (**a**) Mean ± SEM of maximal % aggregation of 70 black and 84 white PRP samples measured by light transmission aggregometry following stimulation with 500 μg ml^{-1} arachidonic acid (AA), 4 μM ADP, anti-CD9 (500, 750 or 2000 ng ml^{-1}), collagen-related peptide (CRP, 10 or 20 ng ml^{-1}), PAR1-AP (1.0 or 2.5 μM), or PAR4-AP (50 or 75 μM PAR4-AP). *, $P < 0.0001$, 2-sided Mann-Whitney for maximal % aggregation. Race was the dominant determinant of the PAR4 ARS when we considered the racial differences in age, gender, body mass index (BMI) and platelet count (P=5.15×10^{-8}, ANOVA partial F statistic of race for PAR ARS, normality of residuals checked by qq-plot). (**b**) Time to 50% aggregation of washed platelets from black (n=5) and white (n=5) subjects after thrombin activation through PAR4 in the presence of 20 μM of the PAR1-specific antagonist, BMS-200261. P=3.56×10^{-5}, partial F statistic for race effect using 2-way ANOVA (concentration, race). Insert demonstrates ability of 20 μM BMS to block maximally stimulated PAR1- but not PAR4-induced platelet aggregation. (**c**) PCA of 2 million genome wide genotype markers demonstrated two groups of PRAX1 subjects that strongly correlated with self-identified race and ethnicity.

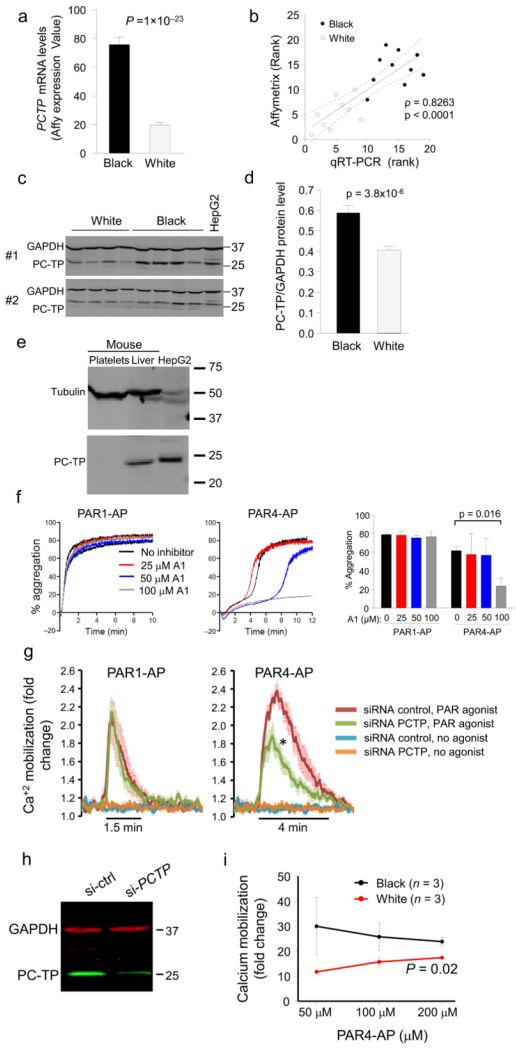

Figure 2.

Racial differences in human platelet PC-TP expression and function. (**a**) Mean *PCTP* mRNA levels are higher in 70 blacks than 84 whites ($P=1\times10^{-23}$, 2-sided t-test). (**b**) PCR validation of *PCTP* mRNA. Correlation between microarray and qRT-PCR data using linear regression (SPSS15.0 software). The regression line with its 95% confidence intervals is shown ($P<0.0001$, Pearson correlation, Fisher's z transform). (**c**) Representative western blots showing higher PC-TP levels in platelets from 8 blacks than 8 whites. PC-TP and GAPDH antibodies probed filters that were transferred from the same gel. Image cropped for clarity. (**d**) Summary of western blot quantification of platelet PC-TP from 70 blacks and 82 whites ($P=3.8\times10^{-6}$, 2 sided t-test). All samples are derived from the same experiment and processed in parallel. (**e**) Western blot of mouse platelet Pctp with controls. Image cropped for clarity. (**f**) Representative aggregometry tracings of platelets in stimulated with PAR1-AP (left) or PAR4-AP (center) with differing concentrations of the PC-TP inhibitor, A1. The bar graph (right) summarizes studies from 3 subjects (2W, 1 B). Compound A1 blocked platelet aggregation to PAR4-AP but not PAR1-AP ($P=0.016$, 2-sided t-test). Color key for panels f-h shown in panel f. (**g**) Calcium mobilization in the megakaryocytic cell line, Meg-01 transfected cells with a control siRNA or an siRNA against PCTP after no agonist or stimulation with PAR1-AP (left) or PAR4-AP (right). Curves represent mean ±

SEM of three separate experiments for PAR1-AP and four different experiments for PAR4-4P. *, (*P*=0.013, 2-sided *t*-test) for the maximum fold change between siRNA-PCTP vs. si-control. (**h**) Immunoblot of Meg-01 cells transfected with non-targeting siRNA control (si-ctrl) or human PCTP siRNA-SMART pool (si-*PCTP*). (**i**) Effect of race on PAR4-AP-mediated platelet calcium mobilization (*P*=0.02, 2-way ANOVA).

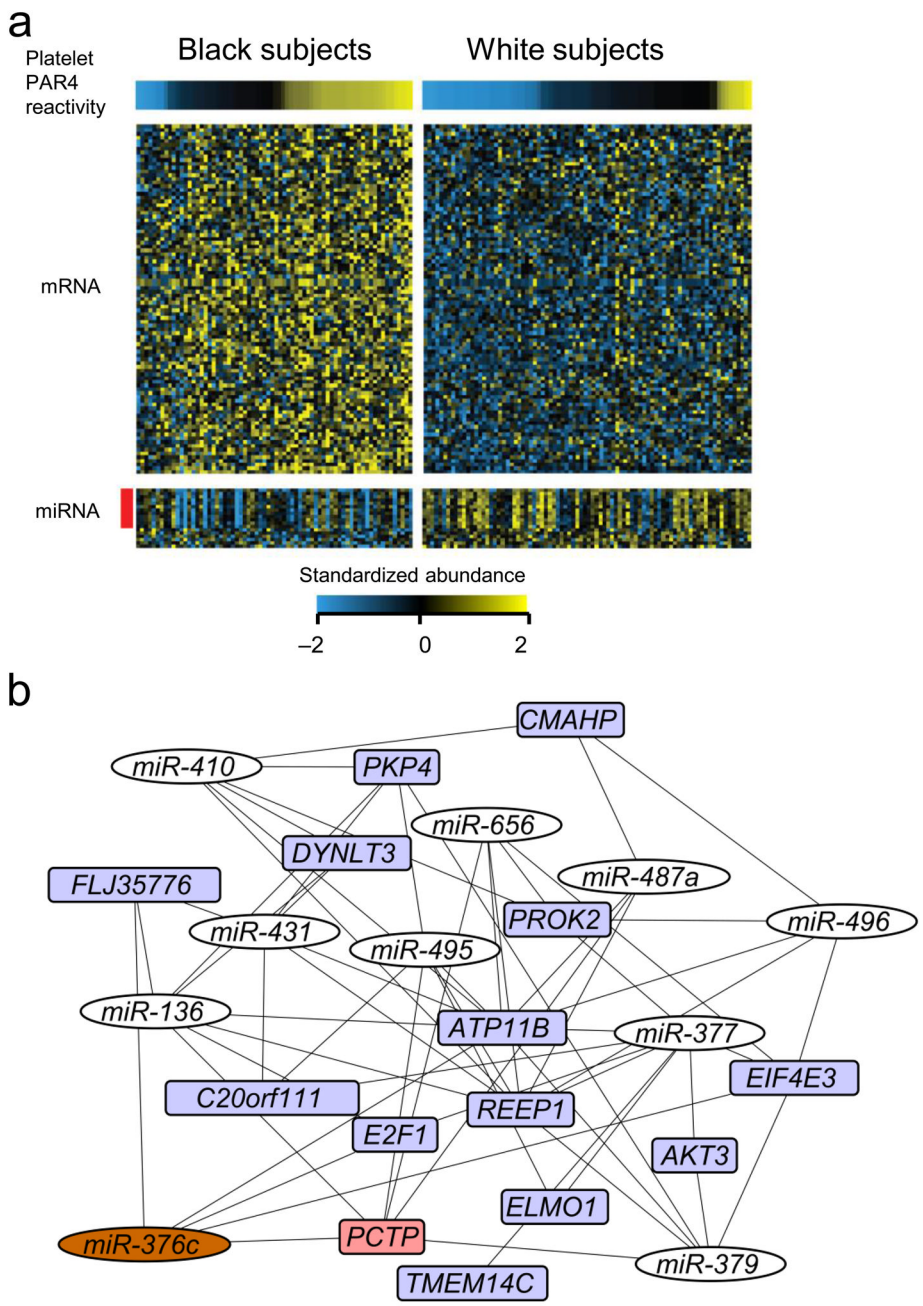

Figure 3.

Relationships among racial differences in PAR4 phenotype and transcripts. (**a**) Racial differences between 70 blacks and 84 whites for PAR4 reactivity, mRNAs and miRNAs are presented in 3 heatmaps. Each column represents data from the same individual. Rows represent 93 mRNAs (in middle panel) and 18 miRNAs (lower panel) that are DE by race and correlated with PAR4 reactivity. Vertical red bar indicates strongly correlated *DLK1-DIO3* region miRNAs. (**b**) Network of miRNA-mRNA pairs that are differentially expressed by race and PAR4-mediated platelet aggregation.

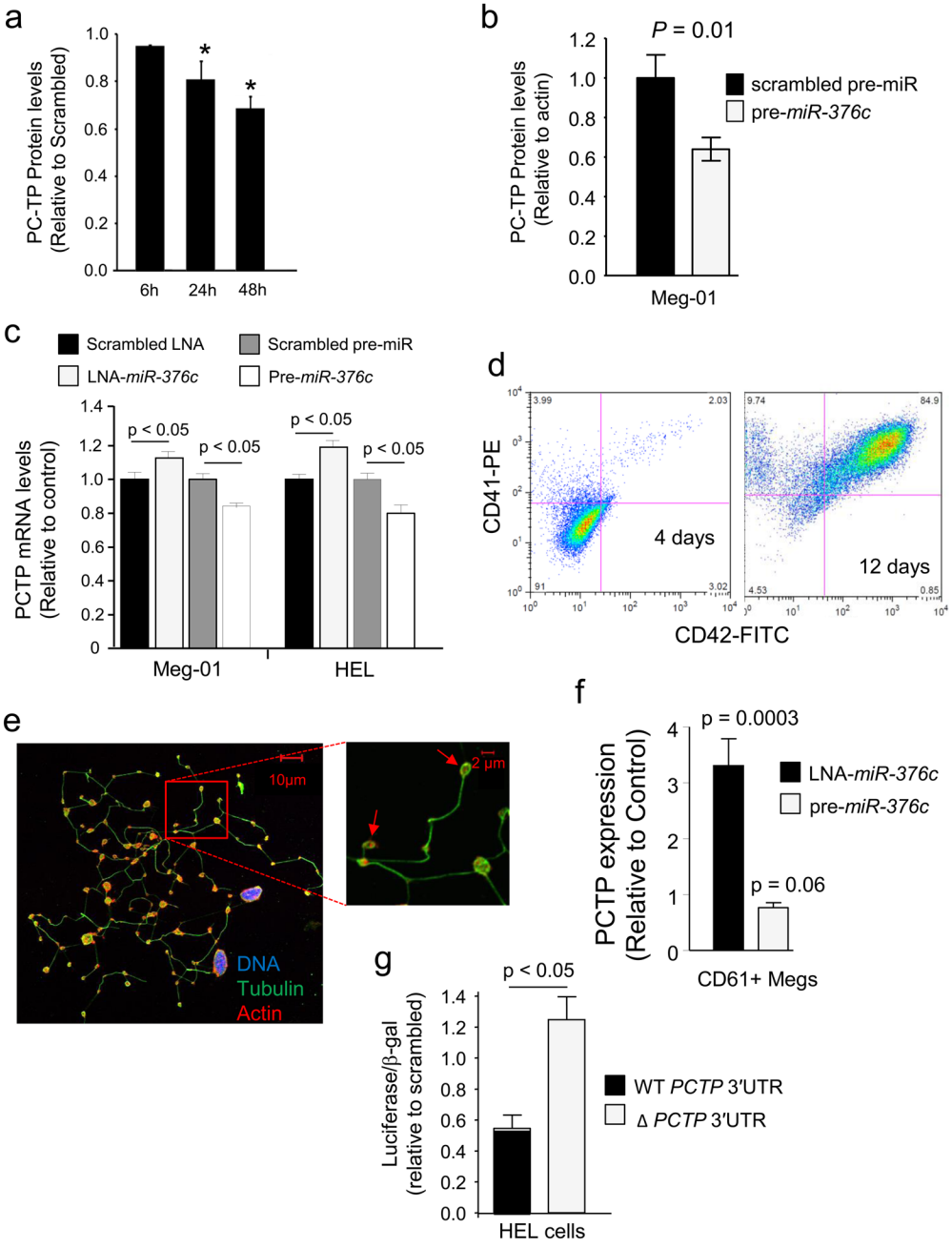

Figure 4.

MiR-376c regulates *PCTP* expression in megakaryocytes. (**a**) Transfection of *miR-376c* diminishes PC-TP protein expression in Dicer-low HCT cells. *, $P<0.05$. (**b**) Transfection of *miR-376c* diminishes PC-TP protein expression in the megakaryocytic cell line, Meg-01. (**c**) Transfection of *miR-376c* locked nucleic acid (LNA) inhibitor increased *PCTP* mRNA levels, while overexpression of the *miR-376c* precursor decreased *PCTP* mRNA in megakaryocytic cell lines, Meg-01 and HEL cells. P-value calculation is T-test relative to appropriate scrambled control. Levels were normalized to β-actin. (**d**) Demonstration of human $CD34^+$ hematopoietic stem cell derived megakaryocytes. Flow cytometric analysis of CD34+ cells cultured for 4 and 12 d and stained for megakaryocyte-specific markers CD41

and CD42. (**e**) 14 d megakaryocytes showing proplatelet formation with DNA (DAPI, purple-blue), α-tubulin (green) and actin (red) staining. (**f**) qRT-PCR quantification of *PCTP* in 14 d CD61+ 14 d megakaryocytes, transfected with *miR-376c* locked nucleic acid (LNA) inhibitor or pre-*miR-376*. Levels were normalized to β-actin. (**g**) Activity of a luciferase reporter with the 3′UTR of *PCTP* with a wild type (WT PCTP) or mutated miR-376c (ΔPCTP) target site. Only the WT construct was knocked down when co-transfected with *miR-376* precursor. Levels were normalized with a β-gal control.

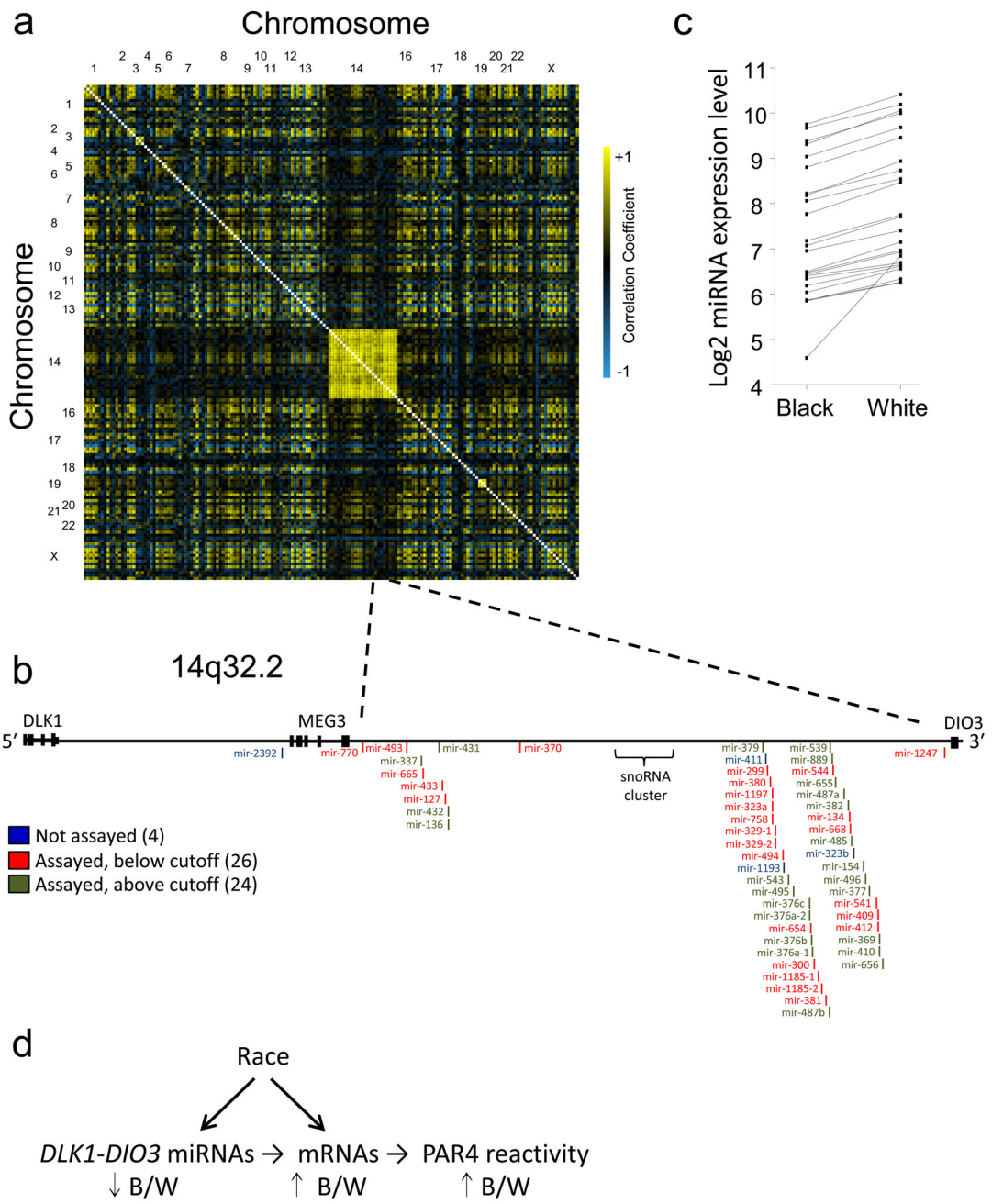

Figure 5.

A large miRNA cluster in the *DLK1-DIO3* region is differentially expressed by race. (**a**) Heatmap of Pearson's correlation coefficients amongst all combinations of pairs of the 178 commonly expressed platelet miRNAs. (**b**) The approximate locations of the 54 miRNAs in the *DLK1-DIO3* region. Color coding indicates which miRNA genes had at least one mature miRNA product detected above or below the arbitrary threshold of commonly expressed miRNAs and the four miRNA genes that were not queried. Figure not to scale. (**c**) Each dot represents the average expression of one of the 24 *DLK1-DIO3* region miRNAs above cutoff. Using a binomial model, the chance that all 24 of the expressed *DLK1-DIO3* miRNAs would be higher in whites is 1.2×10^{-24} based on a genome-wide (excluding the

DLK1-DIO3 region) rate of 51.4% of DE miRNAs higher in whites. (**d**) A schematic summarizing the relationship of race with the expression of the *DLK1-DIO3* region miRNAs and target mRNAs and PAR4 reactivity.

Table 1

miR-376c levels correlated against *PCTP* mRNA levels, PC-TP protein levels, and PAR4 reactivity in all 154 PRAX1 samples.

	Correlation with *miR-376c* levels Pearson r (*P*-value)
***PCTP* mRNA**	−0.214(0.008)
PC-TP protein	−0.211(0.009)
Platelet PAR4 reactivity	−0.161(0.049)